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Carriage of a Single Strain of Nontoxigenic *Corynebacterium diphtheriae* bv. Belfanti (*Corynebacterium belfantii*) in Four Patients with Cystic Fibrosis

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ABSTRACT Cystic fibrosis (CF) patients are commonly colonized by bacterial pathogens, which can induce persistent lung inflammation and may contribute to clinical deterioration. Colonization of CF patients and cross-transmission by *Corynebacterium diphtheriae* have not been reported so far. The aim of this article was to investigate the possibility of a cross-transmission of *C. diphtheriae* biovar Belfanti between four patients of a CF center. *C. diphtheriae* biovar Belfanti (now formally called *C. belfantii*) isolates were collected from four patients in a single CF care center over a period of 6 years and analyzed by microbiological methods and whole-genome sequencing. Epidemiological links among patients were investigated. Ten isolates were collected from 4 patients. Whole-genome sequencing of one isolate from each patient showed that a single strain was shared among them. In addition, one patient was found to have the same strain in two consecutive samplings performed 9 months apart. The strain was nontoxigenic and was susceptible to most antimicrobial agents. Ciprofloxacin resistance was observed in one patient. The idea of transmission of the strain among patients was supported by the occurrence of same-day visits to the CF center. This study demonstrated colonization of CF patients by *C. diphtheriae* biovar Belfanti (*C. belfantii*), and the data suggest persistence and transmission of a unique strain during at least 6 years in a single CF patient care center.

KEYWORDS *Corynebacterium diphtheriae*, colonization, cystic fibrosis, epidemiology, genomic sequencing, transmission

A large fraction of the mortality of cystic fibrosis (CF) patients is attributed to infections of the respiratory tract, which can be caused by multiple pathogens, and cross-transmission within CF centers themselves is an important health care-related issue (1–3). The genus *Corynebacterium* includes a high number of pathogens, most of them being opportunistic (4). So far, only *Corynebacterium pseudodiphtheriticum*, *C. propinquum*, and *C. accolens* have been reported from CF patients (5–7). *C. diphtheriae*, the most pathogenic *Corynebacterium* species that causes diphtheria, has not been reported in paucisymptomatic or asymptomatic CF lung colonization to our knowledge. Typical diphtheria is caused by strains that produce the diphtheria toxin. Although the disease has almost disappeared in countries with high toxoid vaccine coverage, the pathogen still circulates in the human population (8–10). Further, nontoxigenic *C. diphtheriae* strains can be recovered from a variety of infections, including respiratory

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tract infections, skin infections, and bacteremia (11, 12). Four biovars of *C. diphtheriae* are distinguished by biochemical characteristics. Whereas biovars Mitis and Gravis can harbor the diphtheria toxin gene, biovar Belfanti isolates have been described as toxigenic only very rarely and biovar intermedius is rarely isolated (13–15). Recently, *C. diphtheriae* biovar Belfanti isolates were recognized as representing a novel species called *C. belfantii* (16). The aim of this study was to investigate potential cross-transmission within a group of four patients with lower respiratory tract colonization by nontoxigenic *C. diphtheriae*. These patients were followed in a single CF center during a period of 6 years, and our genomic analyses showed that they were colonized by a single *C. diphtheriae* strain.

MATERIALS AND METHODS

Identification of cases. Cases were identified in the regional consultation CF Center of a university hospital between January 2011 and November 2016. During their visits at the center, patients are systematically screened for the presence of opportunistic infectious agents and the evolution of antimicrobial resistance is monitored. The inclusion of cases was performed retrospectively based on at least one sample positive for *C. diphtheriae* upon microbiological screening from sputum or induced sputum. Clinical and laboratory data (sex, age at the time of diagnosis, pulmonary functionality, long-term or sequential antibiotic therapies, symptomatology at the time of diagnosis, respiratory coinfections) were collected for each patient.

Epidemiological investigations. The CF center includes three wards (consultation, pulmonary function testing, and chest physiotherapy) and an imaging department. The timeline of visits of patients to the various wards of the CF center was investigated from their clinical records. Patients underwent chest physiotherapy either before or after their consultation with the physician, and a sputum specimen was systematically collected. Afterward, they were directed to the pulmonary function testing ward. The waiting room is common. All patients wore masks. Patients entered individually in the examination rooms, in which surfaces are cleaned and aeration is performed between patients.

To investigate infection control measures and detect possible factors that might have favored patient-to-patient transmission, all health care workers of the three wards who had worked during the study period were met and interviewed about implementation of standard precautions, material management, and patient care organization. In addition, the health care workers who were in charge of the included patients were screened on a voluntary basis at the time of the study (June to October 2017) for *C. diphtheriae* colonization in the nasopharynx. For this purpose, swabs were plated onto blood agar medium, on which five fosfomycin disks (50 μg/disk) were then deposited. Colonies growing around the disks after 18 to 24 h at 37°C were subcultivated on Tinsdale medium agar and incubated at 37°C.

Bacterial identification and characterization. The isolates were identified as *C. diphtheriae* at the local microbiology laboratory by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) (Bruker) (beginning in September 2016) or by using API Coryne (bioMérieux) (through August 2016). Confirmatory analysis and *tox* gene detection were performed at the National Reference Center. The biovar of isolates was determined based on the combination of nitrate reductase data (positive in biovars Mitis and Gravis, negative in biovar Belfanti) and glycogen fermentation data (positive in biovar Gravis only). Antimicrobial susceptibility was characterized by the disk diffusion method (Bio-Rad, Marnes-la-Coquette, France), and the MIC was determined by the Etest method (bioMérieux, Marcy l'Etoile, France). The sensitivity was interpreted using CA-SFM/EUCAST V2 (September 2018) criteria for *Corynebacterium* (https://www.sfm-microbiologie.org/wp-content/uploads/2018/12/CASFMV2_SEPTEMBRE2018.pdf). Susceptibility was tested for the following antimicrobial agents: vancomycin, kanamycin, gentamicin, penicillin G, oxacillin, amoxicillin, imipenem, cefotaxime, clindamycin, azithromycin, spiramycin, clarithromycin, erythromycin, ciprofloxacin, moxifloxacin, co-trimoxazole, trimethoprim, sulfonamide, pristinamycin, rifampin, tetracycline, and linezolid. Genomic sequencing was performed using a NextSeq 500 instrument (Illumina, San Diego, CA, USA) with a 2 $\times$ 150-nt paired-end protocol on the basis of the use of Nextera XT libraries. Contig sequences were assembled using SPAdes v3.9 (http://cab.spbu.ru/software/spades/). Multilocus sequence typing (MLST) was performed from genomic assemblies using the international nomenclature database webpage (https://pubmlst.org/cdiphtheriae/). Read mapping and calling of high-quality single nucleotide polymorphisms (SNP) were performed as described previously (17) using FRC0074 as the reference genome. The maximum likelihood tree was inferred using IQ-TREE (18) with the evolutionary model K3Pu+F selected by optimizing the BIC criterion. The *p*-distances among genomes were estimated using MASH (19). Identification of *C. belfantii* was performed by genomic comparison with the type strains of *C. belfantii* and *C. diphtheriae* on the basis of the average nucleotide identity metric calculated with JspeciesWS (20) as described previously (16).

Ethical statement. The work was conducted in accordance with local and national regulations, as well as the Helsinki Declaration, and was approved by the local ethics committee (Committee for the Protection of Persons EST I, France).

Data availability. The genomic sequence data generated in this work were submitted to the European Nucleotide Archive and are available from the International Nucleotide Sequence Database Collaboration (NCBI/ENA/DDBJ) databases under project accession number PRJEB28372 and run data accession numbers ERR2757916 to ERR2757921.

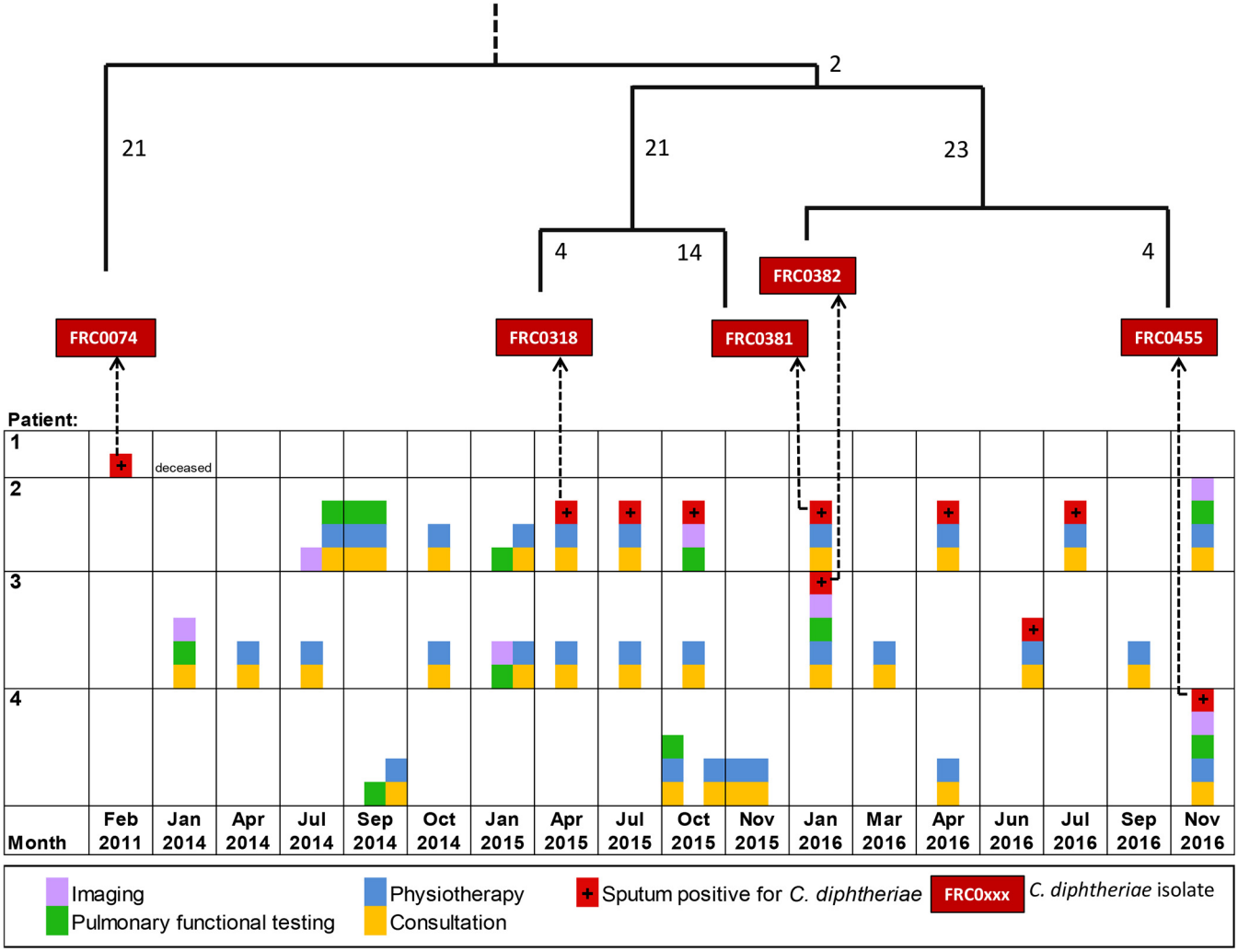

FIG 1 Timeline of events and *C. diphtheriae* detection in four cystic fibrosis patients. Months correspond to different columns in the grid; inside the column corresponding to each month, days (not shown) are distinguished as separate columns. The upper tree recapitulates the phylogenetic relationships among the isolates, which are indicated in red boxes, linked with dotted arrows to their respective patient and isolation time data. The numbers of single nucleotide polymorphisms (SNP) per genome inferred to have occurred are given for each branch of the tree. Jan, January; Feb, February; Mar, March; Apr, April; Jun, June; Jul, July; Sep, September; Oct, October; Nov, November.

RESULTS

Patients. The timeline of visits of patients to the CF center and of detection of *C. diphtheriae* is represented in Fig. 1. The patient characteristics and medical records are summarized in Table 1. From January 2011 to November 2016, four patients of the CF Center had positive screening results for respiratory samples for *C. diphtheriae*. This species was identified from these patients because of the presence of abundant, even though not numerically dominant, coryneform Gram-positive colonies in their oropharyngeal microbiological flora. Cocolonizing bacterial pathogens were found in all patients (Table 1) and were detected repeatedly in the sputum samples. Patient 2 was positive for *C. diphtheriae* for at least 15 months and patient 3 for at least 6 months. Only patients 2 and 4 presented with respiratory exacerbation at the time of *C. diphtheriae* detection and received antibiotics, but they did not require hospitalization. None of the four patients had a dermatological disease or chronic wound. All patients were vaccinated against diphtheria according to French recommendations.

Phenotypic and molecular identification of the isolates. One isolate each from patients 1 (February 2011), 3 (January 2016), and 4 (November 2016) and 2 isolates from patient 2 collected 9 months apart (April 2015, January 2016) were stored and available

TABLE 1 Summary of the medical records of the four patients with positive *C. diphtheriae* isolates in expectoration samples[a]

Patient	Age (yrs)/sex	Baseline lung function (% FEV)	Symptom(s) at the time of positivity	Long-term or recurrent antibiotic therapy	Date of the first positive sample	Other bacteria (coinfection) in the patient's sputum sample(s)	Treatment following positivity	Current status
1	27/F	Noninvasive ventilation, oxygen requirement (30)	Chronic respiratory failure	Amoxicillin, co-trimoxazole for exacerbations	Feb 2011	*Stenotrophomonas maltophilia*, *Haemophilus influenzae*, *Escherichia coli*	No treatment	Deceased in 2013
2	25/M	(106)	Asymptomatic in Apr 2015, exacerbation in Jul 2015	Inhaled tobramycin for 1 mo, Jan 2014; ofloxacin, fusidic acid	Apr 2015	*Staphylococcus aureus*	Fusidic acid, ofloxacin	Alive
3	39/M	(69)	Asymptomatic	Amoxicillin/clavulanic acid, ofloxacin, amikacin for exacerbation	Jan 2016	*Staphylococcus aureusAcinetobacter baumannii*, *Stenotrophomonas maltophilia*	No treatment	Alive
4	23/M	(75)	Exacerbation in Nov 2016	Piperacillin/tazobactam, teicoplanin, imipenem, co-trimoxazole for exacerbation in 2015 and 2016	Nov 2016	*Staphylococcus aureus*, *Achromobacter xylosoxidans*	Amoxicillin/clavulanic acid, co-trimoxazole	Alive

[a]All samples examined were sputum samples. FEV, forced expiratory volume; F, female; M, male; Jan, January; Feb, February; Apr, April; Jul, July; Nov, November.

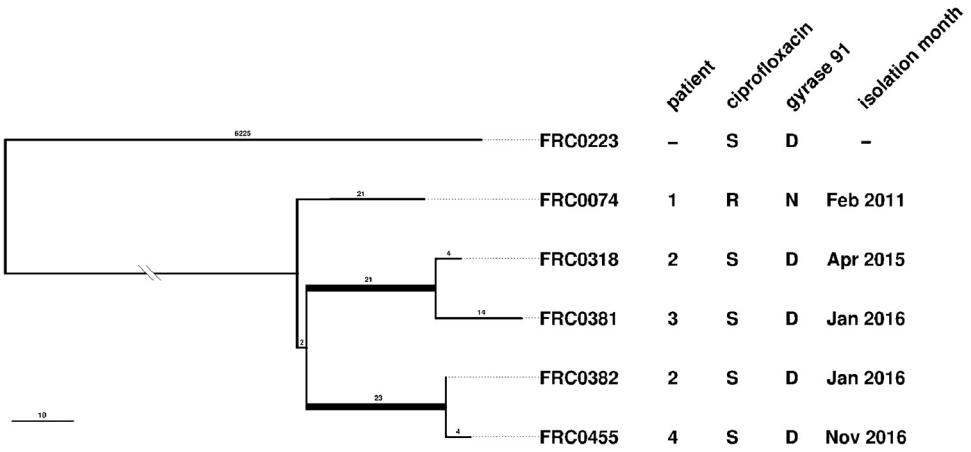

FIG 2 Phylogenetic relationships among the five *C. diphtheriae* isolates. The maximum likelihood tree was rooted with *C. belfantii* isolate FRC0223, isolated in France in 2014 (16). Thick branches represent 100% bootstrap support. The integer value shown above each branch represents the estimated number of single nucleotide polymorphisms (SNP). The scale bar represents 10 SNPs. Columns on the right indicate patient number, isolation month, ciprofloxacin susceptibility, and the amino-acid residue at position 91 of gyrase subunit A (N, asparagine; D, aspartic acid).

for analysis (Fig. 1). The five isolates were identified as *C. diphtheriae*. None of the isolates was toxigenic, and all were of biovar Belfanti. MLST showed that the five isolates belonged to the same sequence type, ST208. Whole-genome sequence variation among the 5 isolates revealed only 62 SNPs among them. The largest SNP distance between two isolates was 58 SNPs and was observed between isolates FRC0074 and FRC0381. Hence, the five isolates were very closely related, showing that they belong to a single strain. In addition, this strain was phylogenetically distantly related (0.49% to 0.77% nucleotide *p*-distances) to all of the other *C. belfantii* isolates available in public repositories and from previous studies (16, 21). One of the most closely related strains (separated from FRC0074 by a *p*-distance of 0.58%) was FRC0223, used as the outgroup in the SNP-based phylogeny of strains (Fig. 2). Phylogenetic analysis based on SNPs (Fig. 2) revealed three subtypes, comprising (i) the isolate from patient 1 (FRC0074); (ii) one isolate from patient 2 (isolate FRC0318) and the isolate from patient 3 (FRC0381); and (iii) the other isolate (FRC0382) from patient 2 and the isolate from patient 4 (FRC0455). Within subtypes, only 18 SNPs separated FRC0318 and FRC0381 and only 4 SNPs separated FRC0382 and FRC0455. Following the recent description of *C. belfantii* (16), the five isolates were reidentified based on their genomic sequence. Their average level of nucleotide identity with the *C. belfantii* type strain (FRC0043^T) was 99.47%, whereas it was only 94.89% with NCTC11397^T, the type strain of *C. diphtheriae*. Therefore, the five isolates belong to the novel species *C. belfantii*.

Antimicrobial susceptibility profiles of the five isolates showed that they were susceptible to all antimicrobial agents, with one remarkable exception: isolate FRC0074 from patient 1 was nonsusceptible to ciprofloxacin. Genomic sequence inspection showed that this isolate had a unique mutation, A to G at position 277 of the *gyrA* gene, coding for subunit A of gyrase, the target of ciprofloxacin. This SNP corresponds to a deduced amino acid change of D to N at protein position 91, which is located within the quinolone resistance-determining region of the gyrase of *Corynebacterium*.

Investigations of possible strain transmission risks. Patient 1 had no recorded contact opportunity with the three other patients at the clinic. In contrast, patients 2 and 3 had visited the CF center the same day on eight occasions for consultations or physiotherapy or for pulmonary function tests between January 2014 and November 2016 (Fig. 1). Further, patient 4 had a consultation and physiotherapy session 30 min after patient 2 in April 2016 and then in November 2016, on the day when he had a positive expectoration sample for *C. diphtheriae*. In addition, patient 4 had a pulmonary function test on the same day as patient 2 in September 2014 and November 2016

(Fig. 1). Therefore, several opportunities for cross-transmission within the CF center were identified among patients 2, 3, and 4, even though no social interaction between CF patients was identified.

Inspections of hygiene measures were retrospectively conducted by the infection control team between June 2016 and June 2017. Local infection control protocols and recommendations regarding health care staff hygiene (mostly with respect to the use of masks, disposable mouthpieces and filters, and specific equipment and hand hygiene) and disinfection of rooms and equipment were correctly observed. However, it was noted that the salbutamol inhalation chamber was disinfected with a low-level disinfectant instead of a mid-level disinfectant as defined following Spaulding's classification (22). Furthermore, after their physiotherapy session, patients did not always wear masks while undergoing pulmonary function testing.

Ten health care workers of the CF center who were regularly in contact with the four patients were retrospectively screened for throat colonization by *C. diphtheriae*. No *Corynebacterium* was isolated in any of the samples from the screened workers.

DISCUSSION

We report four cases of colonization of CF patients by nontoxigenic *C. diphtheriae* biovar Belfanti (now formally called *C. belfantii*). To our knowledge, isolation of *C. diphtheriae* from CF patients was never previously described. Although nontoxigenic isolates can cause a variety of infections, including bacteremia (11), two patients were not symptomatic, whereas the two other had lung exacerbation at the time of first *C. diphtheriae* detection. Other opportunist pathogens of the *Corynebacterium* genus might be involved in CF lung exacerbations (5, 23). In recent years, *C. diphtheriae* became easier to identify by the use of MALDI-TOF. However, *C. diphtheriae* may still be difficult to detect in the CF lungs, given the polymicrobial colonization occurring in most samples, as was observed during this study (Table 1). It is therefore not unlikely that additional cases of colonization might have gone undetected. Likewise, although *C. diphtheriae* was not detected in all sputum samples of the colonized patients, it might have been missed rather than being absent.

Multiple CF patients are typically followed in a given CF center, which creates opportunities for bacterial transmission among patients despite the enforcement of strong infection control measures. A strong suspicion of cross-transmission of *C. diphtheriae* between four patients in our clinic arose given the repeated observation of patients colonized by *C. diphtheriae*. Microbiological investigations fully supported the hypothesis of a single strain being responsible for all of the infections. MLST showed that the five isolates belonged to the same sequence type. The MLST genotype of the isolates, ST208, was not reported previously in the *C. diphtheriae* MLST database and was also never observed previously from another patient in the French national surveillance of *C. diphtheriae*, suggesting that it is not common. Whole-genome sequencing defines the genetic relatedness among *C. diphtheriae* isolates with high precision (24–26). This approach demonstrated that the five isolates belong to the same strain and provided strong support for the hypothesis of cross-transmission among patients and/or contamination from a common source. In addition, the data showed that the strain persisted within patient 2 for at least 9 months. Unfortunately, the additional isolates detected from patient 2 and 3 were not stored.

The SNP variation uncovered by the genomic analysis reflects evolution of the strain since the last common ancestor of the five isolates. Three subtypes were distinguished, two of which comprised isolates from two patients. Subtypes shared by patients may reflect direct transmission between them. Epidemiological investigations support this possibility in one case, as patients 2 and 3 visited the CF center simultaneously on several occasions. However, knowledge on subtype diversity within patients would be required to infer transmission chains with confidence (27). In this study, only one isolate was kept and characterized from each sample. Therefore, one cannot exclude the possibility that subtypes coexisted within single patients, which would lead to the

possibility of the presence of transmission patterns other than those suggested by the phylogeny.

Biovar Belfanti of *C. diphtheriae* (*C. belfantii*) is commonly isolated from the respiratory tract, generally from the nose or throat and often in association with ozaena (28). In contrast, its isolation from skin infections is extremely rare. Therefore, skin wounds of patients or the personnel represent an unlikely reservoir. Transmission by direct respiratory contamination between patients appears to be the most likely transmission route. Transmission of *C. striatum* in an intensive care unit and silent transmission of *C. pseudodiphtheriticum* among CF patients were previously reported (5, 29).

Evolution of antimicrobial resistance occurs frequently in bacterial isolates that colonize CF lungs (30). Our results showed ciprofloxacin resistance in one isolate, whereas the others were susceptible. As no prescription of quinolone antimicrobials was recorded for this patient, one possibility is that the strain had evolved resistance to ciprofloxacin in another ciprofloxacin-treated patient in whom the strain was not detected.

The main limitations of this study were that the infection control investigation was performed retrospectively and that no detailed pattern of transmission could therefore be ascertained. Health care workers or the materials used for patient care may have played the role of vector of *C. diphtheriae* transmission between the patients (31), even though the retrospective screening did not reveal a potential carrier or source of infection. Further, the possibility of hidden patient-to-patient cross-transmission cannot be excluded, as *C. diphtheriae* carriage in some patients may have occurred but gone undetected. Further studies are needed to better define carriage of *C. diphtheriae* by CF patients and to investigate the possible role of patients, health care workers, or environmental sources in cross-transmission. In addition, the clinical significance of nontoxigenic *C. diphtheriae* infections will need to be determined in order to define strategies of treatment, prevention, and control of contamination of CF patients by this bacterial pathogen.

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All of us report no conflicts of interest.

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